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Tetanus Toxin

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INTRODUCTION

The importance of tetanus toxin derives first from the fact that it is capable of producing all the symptoms of clinical tetanus and second from the fact that tetanus is even today a worldwide problem: several hundred thousand people all over the world are affected each year by this toxigenic infection.

In the last two decades, the toxin has been isolated in a homogeneous state (12, 21, 22, 52, 65, 73), and its physical and chemical characteristics have been defined in various laboratories in different parts of the world (5, 12, 14, 20, 22, 50, 52, 63, 64, 73, 83, 85; S. G. Murphy, T. H. Plummer, and K. D. Miller, Fed. Proc., p. 268, 1968). Its structure has been elucidated (20, 50, 64-66), and study of the structure-function relationships has already yielded significant results. Furthermore, the chemical receptor substance in nerve cells responsible for binding tetanus toxin has been identified as a ganglioside (101), and the neural pathway of the toxin from its site of entry or formation in the organism up to its target cells in the central nervous system (CNS) has been clearly demonstrated (28-30, 33-35, 39, 55-57, 82, 87, 94, 108, 111, 112; D. P. Gardner, Ph.D. thesis, University of Tennessee, Memphis, 1972; L. E. King, Ph.D. thesis, University of Tennessee, Memphis, 1970). However, the mechanism of its pathogenesis is still to be elucidated, although it seems that the main pathogenic action of tetanus toxin may be ascribed to its interference with the release of the inhibitory neurotransmitter, presumably glycine. Our inability to understand the precise action of the toxin explains why there is no specific treatment of tetanus and why mortality is still around 50%.

Mass immunization with tetanus toxoid has proved fully effective in protecting populations against tetanus. However, there is a need for a more immunogenic vaccine to be used for single-shot or oral immunization.

In recent years, several reviews have appeared on various aspects of tetanus toxin (3, 31, 38, 42, 58). For this reason, in this review I shall emphasize those aspects which have not previously been fully treated and attempt to clarify those results which, when previously reviewed, gave conflicting evidence. This article will also review the latest developments in the field of the microbiology, chemistry, and pharmacology of the toxin.

TOXIN PRODUCTION

The production of tetanus toxin in high yields has been found to be dependent on several factors, namely, (i) the presence in the culture medium of particular toxigenic factors, (ii) the intrinsic toxigenic capacity of *Clostridium tetani*

strains, and (iii) the mode of cultivation.

Although the toxigenic factors are still to be identified, semisynthetic and synthetic media enabling the production of tetanus toxin to varying degrees have been formulated. Nowadays, the most widely used medium is the one described by Latham and his co-workers (60). More recently, it has been found that *C. tetani* can grow in both liquid and solid forms of a defined and commercially available tissue culture medium, 109 medium, if it is supplemented with cysteine and ascorbic acid. Cultivation of three toxigenic strains in this medium resulted in the production of toxin by two of these (107). Unfortunately, evaluation of the toxin level was not carried out. However, it seems that this medium prepared in solid form could be useful for genetic studies on the toxigenicities of *C. tetani* strains, since the organisms can be subcultured from plate to plate on the same medium. It would also be interesting to verify whether toxigenic strains of *C. tetani* are able to retain their full toxigenic capacity after repeated subculturing on the solid medium, since it has previously been shown that *C. tetani* strains lose their toxigenicity to varying extents after repeated subcultures in liquid medium (74). In fact, it was established that the decrease in the capacity of the organisms to produce the toxin was dependent to some degree on the medium used (76). Toxigenicity was better preserved when the organisms were cultured on semisolid complex medium with glucose than when they were cultured in liquid complex medium or semisynthetic medium (75). According to Nielsen (75), the decreasing ability of toxigenic strains of *C. tetani* to produce the toxin is likely to be related either to a biochemical mutation or to a modification of the cell wall receptor mechanism. The former assumption is supported by the observation that toxin synthesis is influenced by supplementing the culture medium with certain amino acids (99). Subsequently, Mellanby (69) has reported that an excess of glutamate in the medium accelerates the growth of the bacteria and reduces the time necessary for autolysis to start as compared with the time taken in conventional Mueller medium. Since less toxin is synthesized in the presence of excess glutamate, it appears likely that most of the toxin is being produced during the stationary growth phase. In the presence of an excess of glutamate, the change induced in the bacteria, resulting in a shortened stationary phase, could then account for the apparently decreased toxigenic capacity of the *C. tetani* strain.

The critical effect of a change in the metabolism of *C. tetani* cells is further demonstrated by the influence that the mode of cultivation has on toxin yields. *C. tetani* produced less toxin when cultivated in ordinary flasks than when it was cultivated in jars (104, 105). It was also established that growth came to an end about 24 h earlier in jars than in flasks. Lysis also started earlier in jars. Moreover, the pH changes during culture were different: it fell in flasks, and it rose in jars. Since anaerobiosis is less strict in jars than in flasks, contact of the culture surface in jars with oxygen is likely to promote lysis and release of the toxin from the bacterial cells at a time when most of the toxin has already been synthesized. In fact, the toxin was found to remain inside the bacterial cell when the culture was carried out in the presence of nitrogen, whereas it was released and was found extracellularly when oxygen was present. Flask culturing thus seems to better parallel the conditions obtained when culturing in the presence of nitrogen, whereas jar culturing seems to parallel the conditions obtained in the presence of oxygen. An enhancement of toxin synthesis by sweeping the surface of the culture with a stream of air has previously been reported by van Wezel (103). In addition, the fall in pH observed in flasks might also exercise some denaturing effect on the toxin when it is discharged into the surrounding medium, since the toxin is known to be easily and irreversibly denatured at acidic pH values.

MORPHOLOGICAL CORRELATES

In 1966, Lettl and his co-workers postulated that de novo synthesis of toxin in the bacterial cell takes place during the autolysis process (62). Since then, several attempts have been made to correlate de novo synthesis of toxin with structural changes in the bacterial cell.

Electron microscope examination of the ultrastructure of both toxigenic and nontoxigenic *C. tetani* strains at various periods of growth has revealed the presence of intracytoplasmic membranous structures at the beginning of autolysis. Polyribosomes were found to be located close to these structures (78). Pavlova and Sergeeva (78) have suggested that these structural changes may be related to toxin synthesis. However, the fact that these changes were visualized not only in toxin-producing cells but also in dividing and sporeforming cells does not allow us to draw any definite conclusion concerning the relevance of these changes to de novo toxin synthesis. Using a combination of immunological and electron microscope techniques, Volgin and his co-workers (106) have subsequently succeeded in locating the presence of toxin in granules on microgranular osmiophilic masses both outside and inside the lysed cells. However, when we criti-

cally read the reports of these two groups it seems doubtful that the granules seen by Volgin are similar to those described by Pavlova and Sergeeva.

Although direct relationships between the capacities of clostridia to sporulate and their abilities to produce toxin could be established in a few cases, it seems that toxin synthesis in *C. tetani* is inversely related to sporulation (76, 78). In fact, toxigenic strains have been shown to sporulate poorly. Furthermore, the work of Pavlova and Sergeeva (78) strongly suggests that the toxin-producing and sporeforming processes may share a few common steps.

It was also shown that sporulation of the oligosporogenic toxigenic *C. tetani* strain 471 was stimulated when the microorganisms were cultivated in a culture filtrate collected at the beginning of the stationary phase of the periodic culture of the same strain (54). In the latter case it could be assumed that exhaustion of a growth substrate in the culture filtrate would have derepressed the synthesis of sporulation enzymes. Unfortunately, Korovina and her associates (54) did not check whether stimulated sporulation was accompanied by a concomitant decrease in toxin formation. However, these authors did show that outgrowth of the spores gave rise to cells with an unaltered capacity to produce toxin as compared to cells of strain 471.

In connection with these studies it is interesting to consider the results of Smith and MacIver (92), who investigated the growth and toxigenesis of *C. tetani* in vivo. They injected mice with a suspension of *C. tetani* spores in $CaCl_2$ solution and found that within 30 min of injection the spores decreased drastically in number and then outgrew. The mice eventually died. In parallel experiments with the toxin, they measured the time which elapsed between appearance of the first signs of tetanus and death of the animals. These times were similar in mice given 2 minimal lethal doses of toxin and in mice given 120 to 120,000 spores. It is likely that the toxin is produced in the animals injected with spores after 4 to 8 h: tetanus does not develop if penicillin is injected within 4 h after the challenge with the spores (91). However, according to Smith and MacIver (92) it is possible that preformed toxin, rather than newly synthesized toxin, is responsible for tetanus intoxication when this occurs. The existence of preformed toxin inside the spores seems very unlikely and has yet to be experimentally established. The ability of spores to undergo lysis in vivo would also have to be measured.

The nutritional requirements for the germination and outgrowth of *C. tetani* spores were investigated by Shoesmith and Holland (89, 90). It was shown that germination could take place in complex medium under both anaerobic and aerobic conditions, whereas outgrowth required strict anaerobiosis. Moreover, L-methionine, lactate, nicotinamide, and sodium were all necessary for germination in aerobic conditions, whereas only sodium was necessary in anaerobic conditions. Unexpectedly, common spore germinants, such as alanine, inulin, adenosine, and glucose, were found to be without effect in this particular system. In contrast, L-methionine and nicotinamide, which are required for germination of *C. tetani* spores, are not necessary in other bacterial species (89). Subsequently, Shoesmith and Holland (90) reported germination of *C. tetani* spores under anaerobic conditions in a defined medium consisting of leucine, methionine, phenylalanine, lactate, nicotinamide, and sodium ions. The requirement for sodium in aerobic conditions was also confirmed. According to these authors, these more complex germination requirements might be characteristic for *C. tetani* and could, therefore, be used as a taxonomic feature.

The control of tetanus toxin production by genetic factors has not been widely studied. Attempts to correlate the toxigenicity of strains with the presence of a converting bacteriophage have as yet been unsuccessful. Isolation of a *C. tetani* bacteriophage was first reported by Cowles (19). Subsequently, the existence of *C. tetani* bacteriophages was confirmed by the demonstration of mitomycin C-induced lysis of *C. tetani* strains (80, 81). Electron microscope examination of the sediments of both induced and uninduced cultures revealed the presence of phage particles only in the former.

Isolation of a *C. tetani* phage from a garden soil sample has also been reported (86). This phage induced lysis in 11 of the 26 toxigenic strains tested.

Recently, Hara and his co-workers (46) found that bacteriophages from mitomycin C-induced lysates of nontoxigenic *C. tetani* strain derivatives were indistinguishable in size and morphology from that of the toxigenic parent strain. Thus, it does not appear likely that phages are necessary for toxin production (46, 80, 81). However, in the opinion of Hara et al. (46) the question of the possible involvement of a bacteriophage in tetanus toxin production will remain open until a toxigenic *C. tetani* strain "cured" from its prophage has been isolated.

Hara and his co-workers have also reported the isolation of very stable nontoxigenic variants by treatment of an asporogenous, highly toxigenic *C. tetani* strain with various mutagenic agents (46). No revertants to toxigenicity occurred on repeated subcultures of the nontoxi-

genic derivatives. Moreover, it was demonstrated that the nontoxigenic variants produced neither the whole toxin molecule nor fragments of the toxin. These authors put forward the hypothesis that tetanus toxin production could be under the control of a plasmid. However, the possibility that the plasmid may be transduced by the bacteriophage should be considered.

PRODUCTION AND RELEASE

During the exponential growth phase, tetanus toxin is synthesized at a very low rate, most of the toxin being produced after the end of the active growth phase. Coleman (16) investigated the formation of extracellular enzymes in a *Bacillus* sp. He found that their formation occurred almost exclusively after the active growth of the microorganisms had stopped. To account for this fact, he assumed that a competition was likely between increase in cellular material and exoenzyme synthesis at the level of transcription. Subsequently, this assumption was developed into a model applying to those systems in which extracellular proteins are synthesized after the end of the exponential growth phase (17). According to this system, a nutritional limitation at the end of the exponential growth phase would switch off ribosomal ribonucleic acid (RNA) synthesis, resulting in an increase in available RNA polymerase (RNA nucleotidyltransferase). Concomitantly, ribosomal RNA turnover would lead to an increase in RNA precursor pool size, which would permit synthesis of more messenger RNA and thus an increased exoprotein synthesis, provided that the polymerase was not saturated. Coleman and his co-workers (17) used the massive exoprotein production by *Bacillus amyloliquefaciens* as an argument in support of the competition model. In *C. tetani*, the quantity of toxin produced can amount to as much as about 10% of the whole bacterial cell weight (46). In the competition model, the inhibitory effect of excess glutamate on tetanus toxin production (69) could be interpreted as a regulatory effect by amino acid repression similar to that observed on extracellular protease synthesis in *B. subtilis* (68). This model might also explain the above-mentioned stimulation of tetanus toxin production due to addition of the filtrate from a culture undergoing lysis (54) to a fresh culture.

The mechanism of release of the toxin is unknown. Release of the toxin from the bacterial cell (intracellular toxin) into the culture medium (extracellular toxin) has been shown to be accompanied by nicking of the toxin molecule. Intracellular toxin extracted artificially from the washed cells can similarly be nicked into extracellular toxin by mild trypsinization. It has been demonstrated that autolytic enzymes are present in the cell walls of *C. tetani* (98): lysis of *C. tetani* cell walls was found to be accelerated in the presence of trypsin. This raises the possibility that a trypsin-like enzyme might be produced by the bacterial cell, enabling cell autolysis. The same enzyme would also be responsible for the nicking of the released toxin. Korovina and associates (54) showed that the toxin yield of a *C. tetani* culture could be increased by adding, at the beginning of the exponential growth phase, a small amount (1:100) of a filtrate from a culture of the same strain obtained at the time of the decreasing growth phase. These authors ascribed the toxin-promoting action of the filtrate to endogenous metabolites. It could be envisaged that these filtrates might contain either a minute amount of an enzyme necessary for cell wall lysis or inducers for this enzyme, which could be carried over to the developing culture.

However, there is still conflicting evidence concerning proteolytic activity in *C. tetani* strains: some authors found that this species was proteolytic, whereas others did not. This problem has been reexamined recently by Willis and Williams (110) and Williams (109); of the 71 strains tested, none was proteolytic.

Recently, T. B. Helting, V. Neubauer, S. Parschat, and H. Engelhardt (communication to the Int. Conf. Tetanus 5th, Ronneby, Sweden, 1978) have examined *C. tetani* culture filtrates for the protease that is presumably involved in the conversion of intracellular toxin to extracellular toxin. Enzymatic activity was measured with intracellular toxin as a substrate. Weak converting activity was detected in the culture filtrates, but not in the washed viable cells. Enzymatic activity was at its peak at about 96 h. The enzyme exhibited some fibrinolytic activity, but was not itself inhibited by trypsin inhibitor.

The presence of a proteolytic activity in *C. tetani* culture filtrates has previously been considered by Bizzini and co-workers (8, 10). At the time, we observed that pure toxin, in contrast to crude toxin, could be degraded by freezing only if it had previously been incubated with a fresh culture filtrate. We concluded that enzymatic breakdown of one or more peptide bonds must first take place. We also demonstrated that the proteolytic activities in different culture filtrates varied over a wide range.

CHEMISTRY OF THE TOXIN

Our present knowledge of the physicochemical properties of tetanus toxin will be only briefly summarized. I shall attempt instead to clarify previously conflicting results in the light of recent data.

Tetanus toxin can exist as two different molecular forms, designated as intracellular and extracellular toxins. Extracellular toxin differs from intracellular toxin by nicking of a peptide bond. However, the two fragments of nicked toxin are held together by a disulfide bridge. Although the two kinds of toxin show different structures, their physicochemical properties are essentially similar.

Physical and Chemical Properties

Tetanus toxin is a holoprotein with a mean molecular weight of 150,000 (14, 63, 85). Most reports of the amino acid composition have been in fairly good agreement (5, 21, 52, 85; Murphy et al., Fed. Proc., p. 268, 1968). The toxin has been found to contain six free SH groups and two disulfide bridges (5, 20; Murphy et al., Fed. Proc.). However, the presence and nature of amino-terminal groups are controversial. Whereas some investigators failed to detect the presence of amino-terminal groups in both intracellular and extracellular toxins (20; Murphy et al., Fed. Proc.), others identified either leucine or glycine as the single amino-terminal residue (5, 52). More recently, this problem has been reexamined by Helting et al. (communication to the Int. Conf. Tetanus 5th, Ronneby, Sweden, 1978) and J. P. Robinson (personal communication). In intracellular toxin, proline and isoleucine or leucine could be detected. However, it was subsequently demonstrated that a dipeptide, prolyl-isoleucine, had been generated and that proline was the actual single amino-terminal group. In extracellular toxin both proline and leucine were detected (Helting et al., communication to the Int. Conf. Tetanus 5th, Ronneby, Sweden, 1978). Helting and his co-workers (communication to the Int. Conf. Tetanus 5th, Ronneby, Sweden, 1978) proposed that proline represented the amino-terminal group of the light chain and that leucine represented that of the heavy chain.

Structure

Two structural models have been proposed for the toxin. Bizzini and his co-workers (14) investigated toxin derivatives modified in various ways by using the techniques of sodium dodecyl sulfate-gel electrophoresis, gel filtration, and ultracentrifugation. From their results they proposed that tetanus toxin might consist of two identical subunits with molecular weights of 75,000, each subunit being formed from two nonidentical chains with molecular weights of 50,000 and 25,000, respectively, held together by disulfide links (14). This tentative idea of the structure, which had been derived by attempting to reconcile the results obtained by ultracentrifugation in denaturing conditions with those obtained by sodium dodecyl sulfate-gel electrophoresis and gel filtration, has since then been invalidated by the work of other investigators (20, 50, 65, 66). The second structural model was first described by Craven and Dawson (20) and has been confirmed and extended in more recent reports. According to this model, intracellular toxin would consist of a single polypeptide chain with a molecular weight of 150,000, and extracellular toxin would consist of two nonidentical polypeptide chains with molecular weights of 100,000 and 50,000, respectively. Subsequently, Matsuda and Yoneda (65) postulated that extracellular toxin is nicked intracellular toxin. These authors nicked intracellular toxin by mild trypsinization and showed that the two constituent subchains could be separated by gel filtration after reduction of either the nicked intracellular or the extracellular toxin (65). They also succeeded in reconstituting the toxin molecule from the separated constituent subchains (66). This structural model was further supported by the results of Helting and Zwisler, who studied toxin fragments prepared from a papain digest of the toxin (50). Each subchain cross-reacted with the whole toxin, but the subchains did not share antigenic determinants with each other. Table 1 summarizes some of the characteristics of the constituent subchains of tetanus toxin and gives their designations, which differ from author to author.

Each constituent subchain of tetanus toxin has been found to exhibit residual toxicity (65). This toxicity is probably due to contamination of each subchain preparation by a trace of the other, allowing some reconstitution of toxin. Completely nontoxic preparations of each subchain have been isolated by filtration of each subchain on an immunoabsorbent column containing antibodies directed to the other subchain (B. Bizzini, Toxicon **15**:141, 1978).

STRUCTURE-FUNCTION RELATIONSHIPS

Several authors have examined the effects of chemical modifications of particular amino acid residues on the activity of the toxin (7, 11, 13, 36, 85, 93). These results have already been reviewed in detail (3) and will, therefore, only be diagrammatically summarized (Fig. 1). Fragmentation of the toxin molecule has proved to be a very fruitful approach to the investigation of the structure-function relationships in tetanus toxin. Fragmentation has been carried out either by freezing and thawing (6, 8, 9, 15, 79) or by enzymatic degradation (6, 48, 49, 50, 84) of the toxin molecule. The fragments isolated differed in their structures and properties depending on

TABLE 1. *Physical, chemical, and biological characteristics of the constituent subchains of tetanus toxin*[a]

Subchain designation	Mol wt	No. of SH groups	No. of S-S groups	Nature of NH_2-terminal group	Immunological reactivity	Interaction with gangliosides
Light chain (20, 49) or Fragment α (64)	55,000 (20), 46,000–48,000 (49) 53,000 ± 3,000 (64)	3,9 (20)	0 (20)	None (20) Proline (Helting[b])	Cross-reaction with the toxin, not with the heavy chain (64)	Absent (100)
Heavy chain (20, 49) or Fragment β (64)	95,000 (20) 107,000 ± 4,000 (64)	3,7 (20)	0 (20)	None (20) Leucine (Helting[b])	Cross-reaction with the toxin, not with the light chain (64)	Present (100)

[a] Numbers in parentheses correspond to references as given in the text.
[b] Helting et al., communication to the Int. Conf. Tetanus 5th, Ronneby, Sweden, 1978.

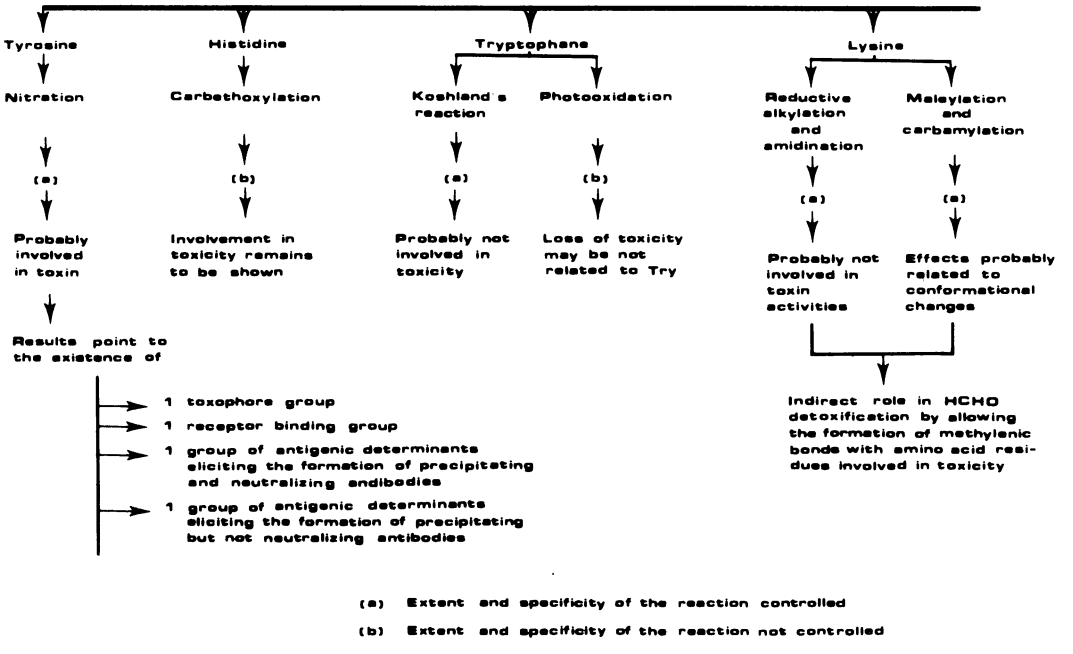

FIG. 1. *Effects of specific modifications of particular amino acid residues on the toxin activities according to the data reported in reference 3.*

the conditions used. The results of these studies are summarized in Table 2.

Such fragments as fraction C and fragment B-II_b are of particular interest, and they are capable of binding to gangliosides (4, 10, 51; Bizzini, Toxicon **15**:141, 1978). Fraction C was shown to be a portion of the heavy chain (50). A fragment similar to fragment B-II_b could also be generated from either the whole toxin molecule or the heavy chain by papain digestion (Bizzini, Toxicon **15**:141, 1978) under the conditions described by Bizzini and Raynaud (6). It therefore seems likely that these fragments contain the portion of the heavy chain which is involved in the binding of the toxin molecule to its receptors in the nerve cell.

INTERACTION OF TETANUS TOXIN AND ITS FRAGMENTS WITH GANGLIOSIDES, ISOLATED PLASMA MEMBRANES, AND CELLS IN CULTURE

Interaction with Gangliosides and Isolated Plasma Membranes

The optimal interaction of tetanus toxin with di- and trisialogangliosides (GD_{1b} and GT1) was first discovered in 1961 (101). The insoluble com-

TABLE 2. *Designations and some characteristics of tetanus toxin subproducts*

Prepn method	Subproduct designation	Mol wt	Toxicity	Interaction with gangliosides	Cross-reaction with:			Reference(s)
					Toxin	Fragment α	Fragment β	
Papain digestion of toxin	B	95,000 ± 5%	Very low	Absent	+	+	+	47, 50
	C	47,000 ± 5%	No	Present	+	0	+	48–50
Trypsin or papain digestion of fragment β	β-1 (trypsin)	ND[a]	ND	ND	+	0	+	67
	α-β-2				+	+	+	
	β-1-PA (papain)				+	0	+	
Papain digestion of toxin	II	56,000–62,000	No	ND	+	ND	ND	84
Freezing and thawing of crude toxin	A-I	98,000	High	ND	+	ND	ND	4, 6, 8–10; Bizzini, Toxicon 15: 141, 1978
	A-II	27,000	Low		+	ND	ND	
	B-I	113,000	Very high		Identity	ND	ND	
	B-II	33,000	Low		+	ND	ND	
	B-II_b	46,000	Very low or no	Present	+	0	+	
Papain digestion of toxin	I_{hc}	100,000	Very low	Absent	+	+	+	In preparation
	II_c	46,000	No	Present	+	0	+	

[a] ND, Not determined.

plexes formed between gangliosides and cerebrosides, and possibly phospholipids and cholesterol, have been assumed to be part of the toxin receptor in nerve cells (102). However, Dimpfel and his co-workers (26), using cell cultures, found that cerebrosides were not crucial for toxin binding. Binding of tetanus toxin to crude synaptic membranes (4) and synaptosomes (37, 40, 70) isolated from rat spinal cords has also been reported. In addition, synaptosomal membranes (37, 59) and neurons (23, 45) were shown to be enriched in gangliosides, such as GD_{1b} and GT1.

Recently, van Heyningen (100) investigated the fixation of these gangliosides by the isolated subchains of the toxin. He demonstrated convincingly that the heavy chain bound gangliosides, whereas the light chain did not, and he proposed that the ganglioside-binding site on the toxin molecule was located on the heavy chain. He also speculated that it is the light chain that exercises the toxic activity, since the heavy chain is involved in binding. This situation has been shown to be true for other toxins, such as diphtheria and cholera toxins, in which one of the subchains is involved in binding and the other subchain is involved in biological activity. More recently, van Heyningen's data have been confirmed by other investigators (51; Bizzini, Toxicon 15:141, 1978).

Fraction C (50) and fragment B-II_b (Bizzini, Toxicon 15:141, 1978) have been shown to represent a portion of the heavy chain. The B-II_b fragment was found to fix both to gangliosides and to crude synaptic membranes isolated from rat spinal cords (4). Helting and his co-workers (51), who developed an original method involving radioactively labeled gangliosides to study the interaction of tetanus toxin and its fragments with gangliosides, observed weak binding with fraction C if this was added in high concentrations. Antibodies directed to fraction C were found to be as effective in inhibiting the binding of tetanus toxin to gangliosides as was the tetanus antiserum.

Gangliosides present on thyroid plasma membranes are integral parts of the cell receptor for thyrotropin (TSH). This observation and the fact that tetanus can be accompanied by a state clinically resembling thyroid storm prompted Ledley and his associates (61) to investigate the fixation of tetanus toxin to thyroid plasma membranes. Binding of tetanus toxin was found to be analogous to that of [^{125}I]TSH; in addition, binding of ^{125}I-labeled toxin could be either prevented or reversed by either unlabeled toxin or TSH. Binding of TSH to the same membranes was impeded or reversed by unlabeled tetanus toxin. Fixation of tetanus toxin could also be blocked by preincubation of the toxin with its antitoxin, and bound toxin could be partially displaced by excess antitoxin.

In parallel experiments, Habig and his coworkers (44) have used thyroid plasma membranes isolated from defective 1–8 thyroid tumor cells. These cells, which are unresponsive to TSH, were assumed to have either a nonfunctional or a nonexistent TSH receptor as their genetic defect. Moreover, this genetic defect has been shown to be related to the absence of

gangliosides, such as GD_{1b} and GT1. Compared with normal thyroid membranes, the defective membranes showed a drastically reduced capacity to bind tetanus toxin.

These data strongly suggest that the receptor for tetanus toxin in nerve cells may be structurally analogous to the receptor for TSH on thyroid cells. This view is further supported by the observation that tetanus toxin is capable of significantly increasing radioiodine levels in mice injected subcutaneously with 1 minimal lethal dose of the toxin.

Interaction with Cells in Culture

In recent years, much work has been devoted to developing the use of tetanus toxin as a specific neuronal cell marker. Dimpfel and his associates (26) found that primary cultures derived from embryonic CNS bound tetanus toxin, whereas none of the tested cell lines did so. These authors further demonstrated that living cells bound tetanus toxin by their gangliosides. The work carried out by Dimpfel's group has been extended and specified by Mirsky and his co-workers (71). They reported that nonneuronal cells, in contrast to neuronal cells, were not "stained" by tetanus toxin. Their results also showed that binding of tetanus toxin by neuronal cells is likely to represent a general property of all neurons, since neurons from all parts of the nervous system bound the toxin. The differential binding of the toxin to particular neurons in vivo would thus be a matter of accessibility to these neurons. Toxin binding was shown to be ganglioside dependent. In cell cultures at least, gangliosides (GD_{1b}, GT1) would be expressed on the surfaces of neuronal cells but not on those of other cell types. Tetanus toxin can therefore act as a specific surface marker. Such a marker has a great advantage over cytoplasmic markers in that it lends itself to the recognition of live cells. Its use should aid in selection of single cells for primary cultures.

The recent work by Zimmerman and her associate (113; J. M. Zimmerman, Ph.D. thesis, University of Geneva, Geneva, Switzerland, 1977) has made a notable contribution to our understanding of the mode of binding of tetanus toxin to nerve cells. These authors selected mouse neuroblastoma cells for their study, since the capacity of these cells to elaborate neurotransmitters, to generate action potentials in response to electrical stimulation in vitro, and to produce neuron-like processes in culture after reduction of the serum level makes these ideal "laboratory animals" on which to study a neurotropic toxin. They studied the effects of neuraminidase, β-galactosidase, poly-D-glutamate (activator of pinocytosis), and ammonium chloride (solubilizer of cyclic adenosine 3',5'-monophosphate-independent protein kinases) on the binding of tetanus toxin to these cells. They also studied the effects of the binding process on some cultural features of both differentiating and growing cells (see Mode of Action). The results led them to propose the existence of two types of binding: an effective binding and an ineffective one. They further suggested that ineffective binding should be considered as that which resulted in no visible biological effect, whereas effective binding would be associated with a visible biological effect. Tetanus toxin can bind to either of the binding sites, but not to both simultaneously. This fact establishes that there are at least two separate and different sites situated on the toxin molecule such that the binding of one to its type of cell receptor (effective binding) excludes the binding of the other toxin site to its type of cell receptor (ineffective binding). Which site on the toxin molecule will bind to a given receptor will be determined by one or more of several factors: (i) the concentration and distribution of the membrane receptors, (ii) the probability of a "hit" by the particular toxin site, and (iii) the relative affinity of each toxin site for its corresponding membrane receptor. Ineffective binding was shown to be sensitive to both neuraminidase and β-galactosidase and therefore to be ganglioside dependent. In contrast, effective binding was found to be ganglioside independent.

However, further investigation of the toxin binding, using ^{125}I-labeled toxin, showed that the situation was more complicated (Zimmerman, Ph.D. thesis). In growth cultures of neuroblastoma cells obtained in the presence of serum, binding was found to be ineffective, since it produced no visible biological effect. In addition, ineffective binding was shown to exhibit two levels of affinity. At one affinity level, binding of labeled toxin could be reversed by unlabeled toxin, whereas at the other level it could not. Binding which is displaceable is by definition specific, whereas nondisplaceable binding is "nonspecific." It was found that 53% of bound toxin was displaceable and 47% was not.

In contrast, in differentiating cultures obtained in the absence of serum, binding was shown to be effective, since it resulted in visible biological effects. With differentiating cultures, three distinct classes of receptors were found to be involved in toxin binding. With the first class of receptors, binding was dependent on the presence of *N*-acetylneuraminic acid residues (the toxin was unbound by neuraminidase). With the second class, binding was dependent on the presence of β-galactoside links (the toxin was unbound by β-galactosidase). With the third class

of receptors, binding was both neuraminidase and β-galactosidase independent. The first class of receptors, which was further subdivided into receptors from which bound labeled toxin could be displaced by unlabeled toxin and receptors from which bound toxin could not be displaced, proved to be identical to the class of receptors on growing cells involved in ineffective binding. In contrast, receptors belonging to the second and third classes were found to be involved in effective binding. Moreover, the latter two classes of receptors accounted for 46% of the total binding.

According to the hypothesis of Zimmerman and Piffaretti (113), effective binding of the toxin would be followed by internalization of the toxin and its transportation to its specific site of action. The toxin's effect would therefore be directly related to the amount of toxin bound effectively. Furthermore, the effective binding would result in blockage of the ineffective-site-bound toxin, which would then be slowly degraded by lysosomal enzymes. Only a part of the effective-site-bound toxin was found to be internalized by pinocytosis, and it was proposed that at least one other mechanism was operating.

Although the question can be raised at this point as to whether neuroblastoma cells do actually substitute for neuronal cells, the results reported here seem to indicate that neuroblastoma cells are an appropriate tool for investigating the interaction of tetanus toxin with cell membranes. In fact, it has been shown that binding of tetanus toxin by cells other than nerve cells was negligible (Zimmerman, Ph.D. thesis).

The putative existence of ineffective and effective bindings of tetanus toxin seems to be strengthened by the fact that it provides us with a base for explaining some unexpected results found in the literature.

Tetanus toxin has been shown to be present in the CNS before the onset of the first symptoms of tetanus and to still be detectable there several weeks after cessation of the symptoms (35). The toxin detected by Habermann (35) could then be that toxin which would have been bound ineffectively and is, therefore, devoid of biological effect.

Tetanus toxin has also been found to interact with neuraminidase-sensitive structures present both on synaptosomes (E. Habermann, unpublished data, cited in reference 41) and in primary cultures of nerve cells (25). In fact, it was verified that neuraminidase was without influence on the lethality of the toxin injected simultaneously either intravenously or intramuscularly (41). This result could be expected if the biologically active toxin is the one which is bound effectively, namely, the one bound to neuraminidase-insensitive receptors.

Retrograde intra-axonal transport of tetanus toxin has been soundly established. It has been postulated that the transport may depend on binding of the toxin to gangliosides of the neuronal membrane (94). However, by preincubating the toxin with gangliosides, Stöckel and his colleagues (95) failed to inhibit the transport by more than a maximum of about 40% (95). Subsequently, Habermann and Erdmann (41) reported that neuraminidase injected simultaneously with the toxin was unable to diminish the ascent of tetanus toxin. Again, the existence of ganglioside-independent receptors for the toxin would provide an explanation for the limited capacity of neuraminidase and gangliosides to interrupt the neural ascent of toxin.

MODE OF SPREAD

The studies on the neural ascent of tetanus toxin have already been reviewed extensively (3, 31), and these are, therefore, only given diagrammatically (Fig. 2). Some data relating to the fate of the toxin in vivo are presented in Fig. 2.

Recent work by Green and her associates (32) has shown that transport of tetanus toxin in the ventral roots was exclusively along α and not γ fibers. Therefore, accumulation of the toxin was found to occur selectively in α motor neurons, γ axons remaining essentially free of toxin.

Schwab and Thoenen (88) made a detailed study of the uptake of colloidal gold particles coated with tetanus toxin by nerve terminals in rat irises. They observed that the particles were selectively bound to the membranes of autonomic nerve terminals and preterminal axons. The toxin-gold complexes were found to be preferentially located in membrane-bounded smooth endoplasmic reticulum-like compartments either as single grains or in rows and clusters. At 14 to 16 h after injection, gold label was detected in the superior cervical ganglion. These investigators suggested that specific binding of the toxin to its receptors on the nerve terminal membrane would be likely to trigger the removal, by internalization, of the occupied receptors together with the toxin and subsequent retrograde transport of the latter.

It is known that molecules are transported from their somal sites of synthesis along the axons down to the axon terminals (orthograde transport), securing their replenishment. In the case of tetanus toxin, on the other hand, the molecules are transported by the axoplasmic route centripetally from the peripheral nerve endings up to the CNS along the axons (retrograde transport). In addition, tetanus toxin has

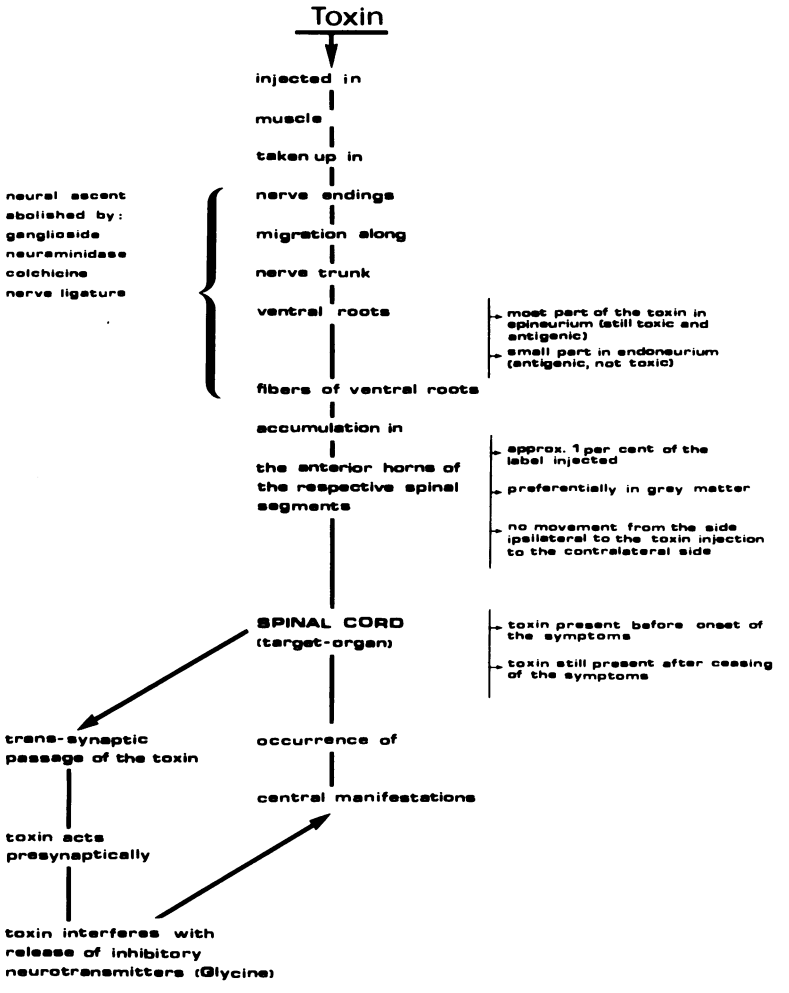

FIG. 2. *Mode of spread and of action of tetanus toxin diagrammatically depicted from the data reported in reference 3.*

been shown to mainly move intra-axonally and not through intraneural, extra-axonal tissue spaces (retrograde intra-axonal transport).

Nature of the Label Present in the Central Nervous System

At this point, a fundamental question to be raised is whether the label measured in vivo actually represents the original toxin or a toxin metabolite (31). With a view to answering this question, Habermann and his co-workers (43) investigated the label extracted in denaturing and nondenaturing conditions from the spinal cords of rats and cats intoxicated with ^{125}I-labeled toxin. These authors showed that most of the label extracted was indistinguishable on gel filtration from tetanus toxin, but a small part of the label exhibited the pattern of a compound with a molecular weight in the range of 40,000. Moreover, 85% of the radioactivity extracted was found to be bound specifically to insoluble antibodies and to show a reaction of identity with both labeled and unlabeled toxins. Toxicity ratios of the spinal cord extracts were also shown to be in the range of that of the injected ^{125}I-labeled toxin.

Although it appears that most of the toxin in the spinal cord is likely to be present as intact toxin, the occurrence in vivo of some degradation cannot be excluded, since a fraction of the toxin was recovered as a compound of lower molecular weight. In the view of Habermann and his col-

leagues (43), the question as to whether the toxicity is expressed by the toxin molecule as a whole or after some metabolic modification remains open.

Binding Properties of Toxin Fragments

It has been assumed that transport of the toxin may depend on its binding to gangliosides of the neuronal membrane (94). It seemed likely that toxin fragments which had retained the capacity to bind to gangliosides would also be transported retrogradely, in the same way as the whole toxin molecule. Convincing evidence has been provided in support of this assumption by Bizzini and his associates (10), who demonstrated the uptake of fragment B-II_b by the terminals of various peripheral neurons and its subsequent retrograde transport. These authors suggested the potential use of such fragments as specific carriers for drugs to the nervous system.

Recently, M. Dumas, M. E. Schwab, and H. Thoenen (manuscript in preparation) investigated the characteristics of the retrograde axonal transport of various compounds, together with those of fragment B-II_b. Fragment B-II_b exhibited positive cooperativity, a characteristic not observed with other compounds (M. E. Schwab, personal communication).

MODE OF ACTION

Despite the increasing number of investigations over the last decades which have been devoted to elucidating the mechanism of action of tetanus toxin, it remains unknown. In the first stage, research has aimed at characterizing the electrophysiological disturbances brought about by the toxin in the CNS. In a second stage, much effort was expended to correlate these changes with either morphological or biochemical changes, or both, in the CNS. A few reviews

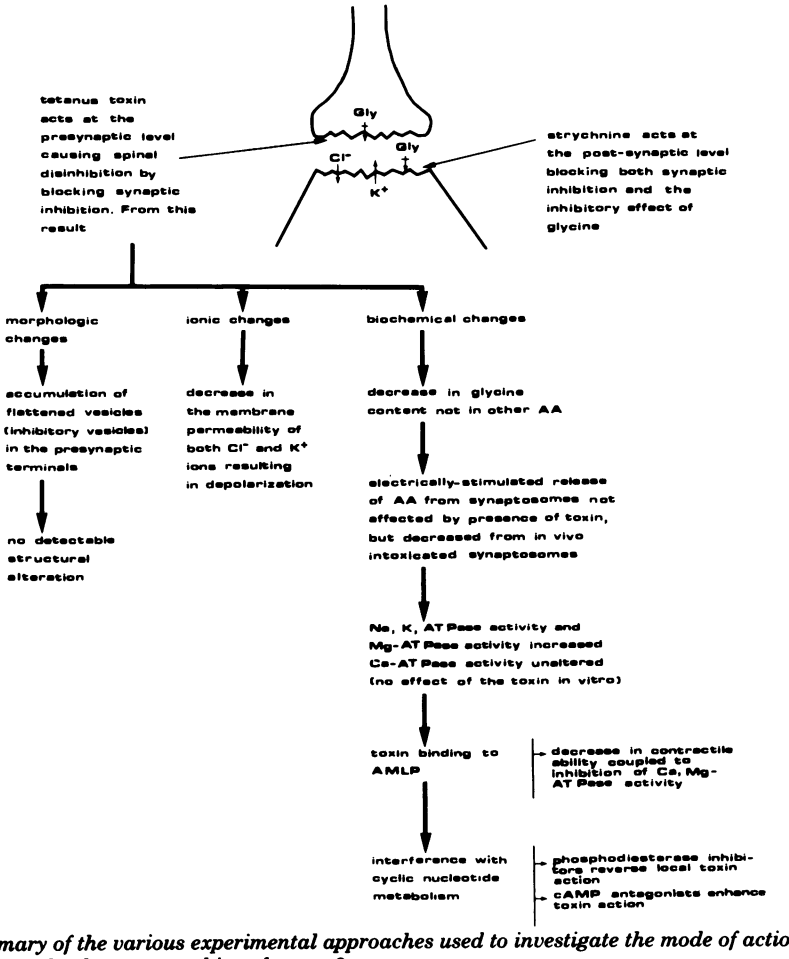

FIG. 3. *Summary of the various experimental approaches used to investigate the mode of action of tetanus toxin according to the data reported in reference 3.*

have appeared on this topic (3, 42, 58). The main results obtained up to 1977 are summarized in Fig. 3.

Recent work has shed light on particular aspects of the pharmacokinetics of tetanus toxin.

Action at the Cellular Level

The demonstration that tetanus toxin can interact with nerve cells in primary cultures (26, 71, 113; Zimmerman, Ph.D. thesis) has permitted study of the effect of the toxin at the cellular level.

In particular, the work of Zimmerman (Ph.D. thesis) has shown that cells grown in the presence of fetal calf serum apparently were not affected by the toxin. In contrast, differentiating cells (grown in the absence of serum) responded to the action of the toxin by a reversal of morphological differentiation visualized by the shortening of processes together with a loss of adherence of the cells to the glass. However, viability of the cells was not significantly altered. In addition, it was shown that the axons induced upon the action of aminopterin and cyclic dibutyryl adenosine monophosphate were insensitive to tetanus toxin. To account for this lack of effect, Zimmerman postulated that the toxin was bound ineffectively to these cells. Furthermore, it appeared likely that the toxin action was not related to that of either colchicine or cytochalasin B, since these compounds have an inhibitory effect on cyclic dibutyryl adenosine monophosphate-induced cells.

Long and short processes turned out to be different in their sensitivities to the toxin action. According to Zimmerman, this could be explained by assuming that the toxin may interfere with either of two messages given by the cell, one of which triggers process formation and the other of which triggers its extension.

Peripheral Action

Demonstration of peripheral paralysis as a result of toxin action has previously been made by several investigators (2, 24, 72). In very elegant experiments (2) with an isolated cholinergic system in rat CNS, primary cultures permitting them to follow the uptake of [^{3}H]choline, its subsequent transformation into acetylcholine, and the release of acetylcholine upon K^+ depolarization (1), Bigalke and his co-workers demonstrated that acetylcholine synthesis was inhibited by tetanus toxin. However, the activity of choline acetyltransferase was not affected by the toxin. The inhibitory action of tetanus toxin on choline uptake was assumed to be due to an indirect "stabilizing" effect of the toxin on the intracellular pool of acetylcholine, preventing its release from the cell. Paralysis as a result of tetanus toxin action has also been reported to occur when the toxin was prevented from accumulating in the spinal cord (2, 72). This phenomenon might also account for the flaccid paralysis caused in animals by the injection of low-nitrated toxin derivatives, bearing in mind that these derivatives have lost their capacity to bind to gangliosides (13). From comparison of the actions of tetanus toxin and type A botulinum neurotoxin, using the above cholinergic system, Bigalke and his co-workers (2) concluded that the two toxins differed in their effects only on a quantitative basis.

Generalized Tetanus

When tetanus toxin is injected intravenously, generalized tetanus will develop. Histoautoradiographic studies on the CNS of rats injected intravenously with ^{125}I-labeled toxin have shown that generalized tetanus was likely to consist of multiple local tetanuses (27).

Toxic Site

A fundamental question that has not yet been answered is whether the toxicity is conveyed by the whole toxin molecule, by one of the constituent subchains, or by a metabolic derivative of the toxin. By analogy to other toxins in which one of the subchains is involved in binding and the other subchain is involved in toxicity, it has been postulated that the light chain of tetanus toxin might be responsible for toxicity (100). Experiments in which the fate of the toxin was followed after its accumulation in the spinal cord have shown that the quasi totality of the toxin present at this level was indistinguishable from the intact molecule. However, the identification of a small amount of a toxin derivative with a molecular weight of 40,000 is consistent with some degradation of the molecule in vivo (43).

This finding should be related to the demonstration of some toxicity for fragment B, consisting of the association of the light chain with the portion of the heavy chain complementary to fraction C (47). In fact, fragment B was shown to cause symptoms of poisoning quite different from those of tetanus intoxication. Furthermore, fragment B proved to be toxic in guinea pigs, less so in mice, and not at all in rats for amounts as large as 200 μg. Death was attributed to a combination of several factors, including asphyxia, cardiac arrest, and general exhaustion.

Autonomic phenomena are well known in severe tetanus (53), and these have been assumed to originate from the overactivity of the sympathetic system (18). It could thus be tempting to ascribe these effects to fragment B or to a frag-

ment of its kind. It can be postulated that only part of the toxin molecules—those bound to the cell membrane—undergo internalization. Furthermore, internalization could concern either the whole toxin molecule or its putative biologically active light chain. Only internalized toxin (or its light chain) would exercise central effects. However, some bound toxin could be cleaved before being internalized, with release of fragment B. Therefore, fragment B would not be internalized, and it could migrate to another target, causing the increased activation of cardiovascular sympathetic mechanisms which have, in fact, been shown to occur early in tetanus (77). Cleavage of the toxin with release of fragment B would be likely to occur during the lag phase existing between the binding of the toxin molecule to the cell membrane and its internalization (Zimmerman, Ph.D. thesis). The postulated cleavage could explain the presence in the CNS of the 40,000-molecular-weight compound detected by Habermann et al. (43). This compound should represent the portion of the heavy chain complementary to fragment B and on which the binding site of the toxin molecule is located.

CONCLUSIONS

Many fundamental problems remain to be solved. One of these is the characterization of the active form of the toxin molecule. Helting and his associates (communication to the Int. Conf. Tetanus 5th, Ronneby, Sweden, 1978) have pointed to the possibility that intracellular toxin, as a single polypeptide chain, may represent a "protoxin" devoid of significant toxicity by itself. The protoxin would be converted into biologically active toxin by nicking of the polypeptide chain upon the action of a bacterial protease. Nicking of the toxin molecule would result in exposure of the heavy chain involved in binding, from which the light chain could then dissociate to exercise its putative biological activity. However, this hypothesis, although interesting, can only be seriously considered after such a protoxin has been isolated.

Whereas the main physicochemical characteristics of the toxin together with its secondary structure are well documented, the primary structure of tetanus toxin is still to be determined. No doubt, comparison of the primary structure of tetanus toxin to those of other nontoxic proteins should help one in understanding the mechanism by which it is toxic as well as the mechanism by which it binds to gangliosides.

However, it appears that the main efforts should be expended to elucidate the mode of action of the toxin, because the ultimate goal of this research is to succeed in developing a specific treatment of tetanus.

The finding that a conjugate consisting of the B-II_b fragment and the light chain of the toxin is still capable of binding to gangliosides and isolated synaptic membranes (Bizzini, Toxicon **15**:141, 1978) has offered a new approach to the investigation of the meaning of the light chain for the biological activity of tetanus toxin.

In addition, advances in the knowledge of the chemistry and pharmacology of the toxin will certainly be fruitful for other fields of research. For example, it is a well-known fact that tetanus toxoid is a potent immunogen. Conjugation of the processed β-subunit of human chorionic gonadotropin with tetanus toxoid has yielded a conjugate in which human chorionic gonadotropin as a self protein was rendered antigenic. This conjugate also proved capable of eliciting the formation of antibodies directed to both human chorionic gonadotropin and tetanus toxoid in humans (97). Conjugates of this kind have permitted both induction of immunity against tetanus and the control of fertility in females (96).

A definite improvement in mass immunization against tetanus will have been attained when a tetanus toxoid effective for oral vaccination is available. It can also be envisaged that such an oral vaccine could also be used for the preparation of a β-human chorionic gonadotropin–tetanus toxoid conjugate, thus offering an alternative to individual birth control in females.

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